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Analysis of the Zidovudine Resistance Mutations T215Y, M41L, and L210W in HIV-1 Reverse Transcriptase

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Although anti-human immunodeficiency virus type 1 (HIV-1) therapies have become more sophisticated and more effective, drug resistance continues to be a major problem. Zidovudine (azidothymidine; AZT) was the first nucleoside reverse transcriptase (RT) inhibitor (NRTI) approved for the treatment of HIV-1 infections and is still being used, particularly in the developing world. This drug targets the conversion of single-stranded RNA to double-stranded DNA by HIV-1 RT. However, resistance to the drug quickly appeared both in viruses replicating in cells in culture and in patients undergoing AZT monotherapy. The primary resistance pathway selects for mutations of T215 that change the threonine to either a tyrosine or a phenylalanine (T215Y/F); this resistance pathway involves an ATP-dependent excision mechanism. The pseudo-sugar ring of AZT lacks a 3′ OH; RT incorporates AZT monophosphate (AZTMP), which blocks the end of the viral DNA primer. AZT-resistant forms of HIV-1 RT use ATP in an excision reaction to unblock the 3′ end of the primer strand, allowing its extension by RT. The T215Y AZT resistance mutation is often accompanied by two other mutations, M41L and L210W. In this study, the roles of these mutations, in combination with T215Y, were examined to determine whether they affect polymerization and excision by HIV-1 RT. The M41L mutation appears to help restore the DNA polymerization activity of RT containing the T215Y mutation and also enhances AZTMP excision. The L210W mutation plays a similar role, but it enhances excision by RTs that carry the T215Y mutation when ATP is present at a low concentration.

Generally speaking, most mutations in HIV-1 reverse transcriptase (RT) have a negative impact on the enzyme; the primary mutations that cause zidovudine (AZT) resistance are not exceptions. In some cases, secondary mutations are selected in viruses that are replicating in patients because these mutations help to compensate for the deleterious effects of primary resistance mutations. In other cases, the most important effect of a secondary mutation is to cause a further decrease in the susceptibility of the virus to the inhibitor. In this report, we have analyzed the effects of the secondary mutations M41L and L210W, in the presence of the primary mutation T215Y, on both ATP-dependent AZTMP excision and polymerase activities of HIV-1 RT to determine whether these secondary mutations enhance the excision of AZTMP by RT and/or act as compensatory mutations that partially restore the reduced polymerase activity of the T215Y mutant.

During the replication of HIV-1, the single-stranded RNA genome is converted into double-stranded DNA by HIV-1 RT. This conversion requires both enzymatic activities of RT: a DNA polymerase that can copy either an RNA or a DNA template and an RNase H that cleaves RNA if, and only if, it is part of an RNA/DNA duplex. Like many DNA polymerases, RT requires both a template and a primer; nucleotides are added sequentially to the 3′ end of the primer strand by the polymerase activity of RT. During normal polymerization, the end of the growing primer strand is at the primer (P) site, and the incoming deoxynucleoside triphosphate (dNTP) binds at the nucleoside (N) site. When the polymerase adds a dinucleotide monophosphate (dNMP) to the 3′ end of the primer strand, inorganic pyrophosphate (PP_i) is released. Immediately after incorporation of a nucleotide, the 3′ end of the primer +1 strand temporarily resides in the N site; translocation moves the end of the primer from the N site to the P site, which allows the cycle to be repeated (1–6).

AZT was the first drug used to treat HIV-1 infections. Although it is no longer the first option for therapy, the WHO still recommends AZT-containing therapies for those who cannot use the first option (tenofovir disoproxil fumarate [TDF] plus lamivudine [3TC] {or emtricitabine [FTC]} plus efavirenz [EFV]) (see http://www.who.int/hiv/pub/guidelines/arv2013/intro/rag/en/index4.html). AZT is a member of the larger class of nucleoside analog reverse transcriptase inhibitors (NRTIs). After being taken up by cells, AZT is converted into the triphosphate form (AZTTP) by cellular kinases. AZTTP is a dNTP analog that binds to the RT/template/primer complex and is incorporated into the growing viral DNA primer by RT. Like other NRTIs, AZT lacks the 3′-OH group needed for the addition of the next dNTP; the incorporation of AZT monophosphate (AZTMP) blocks the synthesis of viral DNA. Early studies identified a number of amino acid substitutions in HIV-1 RT that can contribute to AZT resistance in patients undergoing AZT therapy. These RT mutations include M41L, D67N, K70R, L210W, T215Y/F, and K219Q/E/N (7–10). Most patients treated with AZT who develop resistance have viruses that

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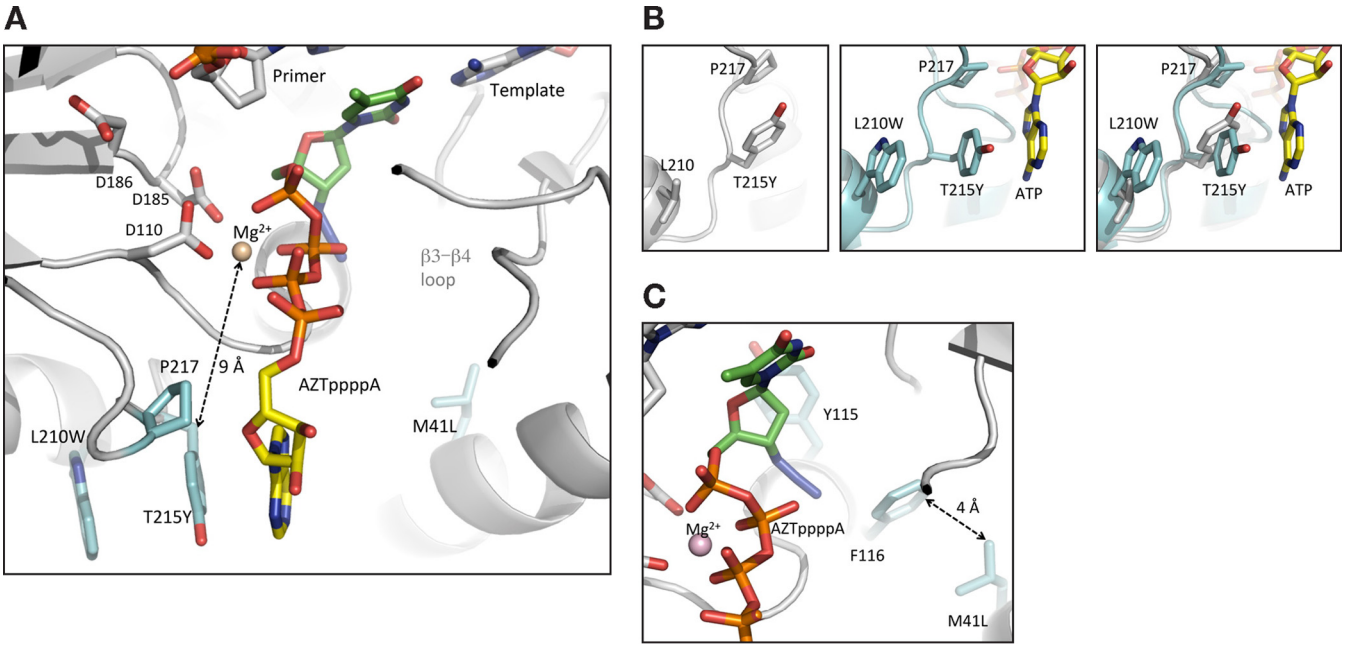

FIG 1 Structure of the region near the HIV-1 RT polymerase active site that is involved in AZT resistance. (A) Structure of the region near the polymerase active site of an AZT-resistant HIV-1 RT (AZT-R) containing the dinucleoside tetraphosphate AZTppppA bound to the RT (2). π-π stacking between Y215 and the adenine ring stabilizes the binding of the ATP moiety to the mutant RT. The AZTMP has been removed from the end of the primer in an ATP-mediated excision reaction, but the reaction product has not yet been released. The L210W and M41L mutations do not appear to make contact with the ATP moiety, suggesting that they may facilitate the excision reaction indirectly. L210W has been modeled into the structure and is located behind the T215Y mutation on the opposite side of the ATP moiety. It is possible that L210W may interact with T215Y to enhance π-π stacking with the adenine ring. The L residue of the M41L mutation is deeper in the RT structure than the WT M residue and does not appear to interact with the substrate directly. (B) Location and orientation of the Y215 side chain in different RT structures. In the apo form of RT, the side chain appears to interact with P217 and is tucked away from the cleft between the finger and thumb subdomains. When the dinucleoside tetraphosphate is bound, the tyrosine side chain (Y215) rotates away from P217 into an orientation that can interact with the ATP moiety. The two structures are compared in the far right diagram. (C) Interactions of M41L with F116. The leucine side chain is far enough away from the dinucleoside tetraphosphate that it is unlikely to interact directly with this substrate. However, the leucine (L41) side chain could interact with F116, which is near the azido group of the AZT moiety. Nonetheless, this interaction does not appear to alter the position of F116.

carry a subset of these AZT resistance mutations; some combinations of these mutations are commonly seen together. One pathway is centered on the T215Y mutation, which is often found in association with the M41L and L210W mutations. The other common pathway involves various combinations of the D67N, K70R, T215F, and K219Q/E/N mutations (11, 12). In this report, we focused on the first set of mutations (T215Y, M41L, and L210W). We are interested in why different secondary mutations are selected in these two pathways.

A number of NRTI resistance mutations are known to act by reducing the binding and incorporation of an NTRI triphosphate (NRTITP) relative to normal dNTPs. This type of mutation (collectively referred to as "exclusion mutations") can be either active, if, for example, there is a direct steric clash between the amino acid substitution and the incoming inhibitor, e.g., when the M184V/I mutations cause a steric clash with the oxathiolane ring of 3TC triphosphate (3TCTP), or passive, in which differences in the interactions of the normal dNTPs and the NRTITPs with the mutant RT favor the incorporation of the normal dNTPs over the analog (e.g., Q151M and K65R) (3–5).

Although some other mechanisms were initially proposed, it is now clear that the most common AZT resistance mutations cause enhanced excision of AZTMP after it has been incorporated into the growing viral DNA (1, 5, 6). The AZTMP moiety is removed from the end of the primer by using an ATP-dependent pyrophosphorolysis mechanism that is related to, but distinct from, polymerization reactions run in reverse (2-4, 9). ATP-dependent excision removes the AZTMP from the 3′ end, freeing the viral primer strand so that DNA synthesis can continue. The amino acid substitution T215F/Y and the accompanying mutations enhance the ability of RT to excise AZTMP. The development of an ATP-excision-dependent AZT resistance mechanism is specific for HIV-1 RT; HIV-2 RT uses a different AZT resistance mechanism (13, 14). Because excision is related to polymerization run in reverse, mutations in HIV-1 RT that affect excision can also affect polymerization; one of the reasons for analyzing these mutations in more detail is to try to better understand how drug resistance mutations affect these two related processes.

Structural and modeling studies have shown that the T215Y mutation is one of two (with K70R) primary AZT resistance mutations (2–5). AZT-resistant HIV-1 RT transfers the AZTMP from the end of the DNA primer to the γ-phosphate of the ATP, forming a dinucleoside tetraphosphate and producing a free 3′-OH group at the end of the primer strand (Fig. 1A) (2–4). There are biochemical data (11, 15, 16), together with the structures of the excision product bound to RTs of the wild type (WT) and the AZT resistance variant that has been designated AZT-R (2), that support the idea that ATP-mediated excision is the mechanism that underlies the common form of AZT resistance. These AZT resistance mutations do not affect the incorporation of AZTTP by

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HIV-1 RT. For the sake of completeness, we have repeated these assays, using both RNA and DNA templates, for the AZT resistance mutations described in this work (see Results). We also point out that due to microscopic reversibility, mutations in RT cannot preferentially speed up the excision of AZTMP from the end of the viral DNA using pyrophosphate as an excision substrate without also causing an equivalent increase in the rate at which AZTTP is incorporated. This means that an excision-based mechanism that uses PP_i as the excision substrate cannot lead to resistance (17, 18).

It has also been suggested that AZT resistance mutations do not primarily affect the binding but rather affect the positioning of ATP (19). That proposal is not supported by either the biochemical data of Dharmasena et al. (16) or the structural data of Tu et al. (2). The aromatic side chain of a Y at position 215 interacts with the adenine moiety of ATP, enhancing the ability of RT to bind the ATP that is used in the excision reaction (Fig. 1A). The location of the tyrosine side chain of T215Y is ~9.0 Å from the polymerase active site (Fig. 1A and B). The other primary AZT resistance mutation in HIV-1 RT, K70R, interacts with the ribose ring and the alpha phosphate of the ATP involved in the excision reaction. A tyrosine at position 215 and an arginine at position 70 specifically interact with a portion of the ATP that does not overlap an incoming dNTP or the pyrophosphate that is produced when a dNMP is incorporated. This is why these mutations, which help HIV-1 RT bind ATP in the excision reaction, can selectively speed up the excision reaction without affecting the incorporation of AZTTP. It is also important to point out that crystal structures show that the WT form of HIV-1 RT does not have any interactions with the ribose ring, the nucleobase, or the alpha phosphate of ATP. Thus, the structural data show how the interactions made by the T215Y and K70R mutations allow AZT-resistant RTs to better bind ATP (2).

Although the role of a Y residue at position 215 is clear, the roles played by the accompanying secondary mutations are far less certain. As mentioned above, M41L is often associated with T215Y, and the addition of the M41L mutation to T215Y reduces the susceptibility of the virus to AZT compared to that with T215Y alone. However, M41L is not in a position where it can interact directly with the ATP that is involved in the excision reaction (Fig. 1A and C) (2), nor is it located near the T215Y mutation; rather, M41 is buried deeper in the RT structure. The leucine side chain of L41 interacts with Phe116 (4.0-Å separation), which is part of the dNTP-binding pocket located near the 3′-OH group of an incoming dNTP (Fig. 1C). The side chain of F116 is also near the azido side chain of an AZTMP on the end of the primer strand, suggesting that the effects of a leucine side chain at position 41 could be transmitted to the AZT group through F116 (2, 20). Because the excision reaction is closely related to the polymerization reaction run in reverse, an incorporated NRTI monophosphate (NRTIMP) must be located at the N site to be excised. The long azido group of AZTMP causes the 3′ end of the primer to preferentially reside at the N site, which makes it a better substrate for excision than other NRTIMPs. It is thought that the M41L mutation may help stabilize the binding of an AZTMP-terminated primer in the N site, facilitating the excision reaction (Fig. 1C). The L210W mutation has not been included in any of the reported structural studies. However, the L210W mutation reduces the susceptibility of HIV to AZT in the presence of the T215Y mutation (7, 21, 22). The indole ring of a tryptophan side chain at W210 has been predicted to be in a position where it could interact with the tyrosine side chain of the T215Y mutant, potentially enhancing the aromatic stacking of Y215 with ATP (22). Based on this logic, the location of L210W has been modeled into the known AZT-R structure in Fig. 1A. It has been suggested that L210W may act as an anchor, preventing any large-scale movements of T215Y that would allow the side chain of Y215 to move away from the ATP-binding site, which could improve ATP binding. T215Y has a negative impact on the polymerase activity of RT. We show here that not only do M41L and L210W improve the ATP-dependent excision reaction, they also, to some degree, compensate for the negative impact of the T215Y mutation on the polymerase activity of RT.

MATERIALS AND METHODS

Preparation of HIV-1 RT. The open reading frames encoding wild-type HIV-1 RT and each of the RT mutants were cloned into a plasmid containing the HIV-1 protease (PR) open reading frame, as previously described (14, 17). The RT coding region was derived from HIV-1 strain BH10 (GenBank locus HIVBH102). The plasmid is based on the expression vector pT5m and was introduced into *Escherichia coli* strain BL21(DE3)(pLysE). Induction with isopropyl-β-D-thiogalactopyranoside (IPTG) (200.0 μl of 1.0 M) causes the bacteria carrying the plasmid to express both RT and PR. After 2.0 h of incubation, approximately half of the RT in the bacteria is converted into the small (p51) subunit by PR. The bacteria were collected by centrifugation, and the cells were frozen at −70°C. The HIV-1 RT heterodimer was purified as previously described (14, 17).

We performed assays to measure the ability of the WT and mutant enzymes to extend a primer on an RNA or a DNA template if the RTs are restricted to a single round of binding and DNA extension (processivity assay) and the ability of the RT to extend a primer if additional rounds of binding and extension are permitted (polymerase assay). Because the mutations can affect the ability of RT to bind the dNTP substrates, we also performed polymerase assays with low concentrations of dNTPs, and we performed kinetic assays to make these comparisons quantitative. Although we did not expect that the AZT resistance mutations, which are in the polymerase domain, would affect RNase H activity, we performed RNase H assays to show that there was no effect. In terms of the effects of the mutations on AZT resistance, we showed that the mutations did not affect the incorporation of AZTTP on either an RNA or a DNA template (NRTITP inhibition assay), and we used excision-extension assays to measure the impact that the mutations had on AZTMP excision at both what is usually considered to be the normal level of ATP in a growing cell (3 mM) and a lower concentration, which might be found in a resting cell (0.6 mM).

Processivity and polymerase assays. The processivity and polymerase assays were previously described (14, 17). In brief, the −47 sequencing primer (5′-CGCCAGGGTTTTCCCAGTCACGAC-3′) (New England BioLabs) was 5′-end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. After purification, the labeled primer was annealed to 1.0 μg of single-stranded M13mp18 DNA for each sample to be assayed by heating and slow cooling. An RNA transcript [generated from a plasmid that contains the HIV-1 PPT, long terminal repeat (LTR), and primer-binding-site sequences (sense strand)] was synthesized by using the T7 MEGAScript kit (Ambion) and a plasmid containing sequences derived from pNL4-3. A synthetic DNA oligonucleotide (Biosource) complementary to the primer-binding-site sequence (5′-GTCCCTGTTCGGGCGCCA-3′) was 5′-end labeled and then annealed to the RNA template.

The RNA template/labeled DNA primer or DNA template/labeled DNA primer combination was resuspended in a mixture containing 25.0 mM Tris-HCl (pH 8.0), 75.0 mM KCl, 8.0 mM $MgCl_2$, 100.0 μg of bovine serum albumin (BSA) per ml, 10.0 mM CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}, and 2.0 mM dithiothrei-

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tol (DTT). One microgram of purified wild-type RT or an RT variant was added to each tube and allowed to bind to the labeled template/primer for 2 min. Extension was initiated by the addition of dNTPs, to give a final concentration of 10.0 μM each. For the processivity assays, 0.5 U of poly(rC)·oligo(dG) per ml was added to the reaction mixtures after the RT-binding step but before the addition of the dNTPs. The polymerase assay mixtures do not contain this "cold trap." Poly(rC)·oligo(dG) prevents RT from rebinding to the labeled primer by binding RT after it disassociates from the labeled template/primer. The reactions were allowed to proceed at 37°C for 10 min and were then halted by the addition of EDTA to the mixtures. The samples were precipitated by the addition of 2 volumes of ethanol. The samples were fractionated by electrophoresis on a 6.0% polyacrylamide gel, and the gel was autoradiographed.

Low-dNTP extension assay. The low-dNTP extension assay was described previously (14, 17). Briefly, the DNA template/DNA primer and RNA template/DNA primer combinations were generated as described above. For each sample, 1.0 μg of wild-type RT or an RT variant was added to the labeled template/primer in a mixture containing 25.0 mM Tris-Cl (pH 8.0), 75.0 mM KCl, 8.0 mM $MgCl_2$, 2.0 mM DTT, 100.0 μg of BSA per ml, and 10.0 mM CHAPS. The reaction mixture was supplemented with 0.1, 0.2, 0.5, or 1.0 μM (each) dATP, dCTP, dGTP, and dTTP. The reactions were allowed to proceed at 37°C for 15 min and were halted by the addition of EDTA to the mixtures. The samples were precipitated by the addition of 2 volumes of ethanol and fractionated by electrophoresis on a 6.0% polyacrylamide gel, and the gel was autoradiographed.

AZTMP excision-extension assay. The ATP-dependent excision-extension assay was done as previously described (14, 17). A synthetic DNA oligonucleotide (5′-CAGGTCACTGTTCGAGCACCA-3′) (Biosource, Camarillo, CA) was 5′-end labeled and then annealed to the DNA template (5′-GCGCAGTGTAGACAATCCCTAGCTATGGTGCTCGAACA GTGACCTG-3′) or the RNA template (5′-GCGCAGUGUAGACAAUC CCTAGCUAUGGUGCUCGAACAGUGACCUG-3′). The 3′ end of the primer was then blocked by the addition of AZTMP, using a purified HIV-1 RT that contains mutations in the RNase H active site (D443A and D549A). The underlined A residues in the sequences of the templates indicate the position in the template that specifies the position at which AZTMP was added to the primer. After purification of the blocked template/primer, the template/primer was incubated with HIV-1 RT or the RT variant in 50.0 μl of a mixture containing 50.0 mM HEPES (pH 7.5); 75.0 mM KCl; 16.0 mM $MgCl_2$; 100.0 μg of bovine serum albumin per ml; 10.0 mM CHAPS; 10.0 μM (each) dATP, dTTP, dGTP, and dCTP; and the indicated concentrations of the various nucleoside triphosphates (NTPs) (ChemCyte) for various lengths of time. The reactions were halted by the addition of EDTA to the mixtures, the salts and nucleosides were removed by passage through a CentriSpin-10 column (Princeton Separations), and the template/primer was precipitated by the addition of ethanol. The products were fractionated on a 15% polyacrylamide sequencing gel. The amount of template/primer (blocked and unextended plus deblocked and extended) and the amount of full-length product (deblocked and extended to the end of the template) were determined by using the PhosphorImager system.

NRTITP inhibition assay. A synthetic DNA oligonucleotide (5′-CAG GTCACTGTTCGAGCACCA-3′) (Biosource, Camarillo, CA) was 5′-end labeled and then annealed to the DNA template (5′-GCGCAGTGTAGA CAATCCCTAGCTATGGTGCTCGAACAGTGACCTG-3′) or the RNA template (5′-GCGCAGUGUAGACAAUCCCTAGCUAUGGUGCUCG AACAGUGACCUG-3′). The annealed template/primer was suspended in 50.0 μl of a mixture containing 25.0 mM Tris-HCl (pH 8.0); 75.0 mM KCl; 8.0 mM $MgCl_2$; 100.0 μg of bovine serum albumin per ml; 10.0 mM CHAPS; and 10.0 μM (each) dATP, dTTP, dGTP, and dCTP and then incubated with 1.0 μg of RT in the presence of various concentrations of the triphosphate form of the analog for 10 min at 37°C. The products were purified and then fractionated on a 15% polyacrylamide sequencing gel. The total amount of template/primer (analog-blocked products and full-length products) and the amount of full-length product (extended to the end of the template) were determined by using the PhosphorImager system. The amount of full-length product in the absence of any NRTITP was considered 100% activity. The activity with the NRTITPs was normalized to this value. The percentage of full-length product was calculated by determining the amount of primer that was extended to the end of the template and dividing this amount by the amount of all of the extension products (full-length and NRTIMP-blocked primers).

RNase H assay. The RNase H assay was previously described (17). The RNA oligonucleotide was obtained from Dharmacon Research, Inc., and has the sequence 5′-GGGCCACUUUUUAAAAGAAAAGGGGGGACU GGAAGGGCUAAUUCACUCAC-3′. This sequence matches the sequence of the HIV-1 RNA genome and extends from just 5′ of the poly(U) tract, through the PPT, and into the U3 region. The RNA oligonucleotide was 5′-end labeled and then annealed to a synthetic DNA oligonucleotide (5′-GAGTGAATTAGCCCTTCCAGTCCC-3′) by heating and slow cooling. The template/primer was adjusted to give a final concentration of 0.2 μM in a mixture containing 25.0 mM Tris-HCl (pH 8.0), 50.0 mM NaCl, 5.0 mM $MgCl_2$, 100.0 μg of bovine serum albumin/ml, 10.0 mM CHAPS, and 1 U of Superasin (Ambion)/μl. The final reaction mixture volume was 12.0 μl. The reactions were initiated by the addition of 35.0 nM RT to the mixtures, and the mixtures were incubated at 37°C. Aliquots of 2.5 μl were removed at the indicated time points, and the reactions were halted by the addition of 4.0 μl gel loading buffer (Ambion) to the mixtures. The reaction products were fractionated on a 15% polyacrylamide sequencing gel. Products were visualized by exposure to X-ray film.

Kinetics. A synthetic DNA oligonucleotide (5′-CAGGTCACTGTTC GAGCACCA-3′) (Biosource, Camarillo, CA) was 5′-end labeled and then annealed to the appropriate DNA template (depending on the dNTP being tested) (5′-GCGCAGTGTAGACAATCCCTAGCTNTGGTGCTCGA ACAGTGACCTG-3′). To allow different dNTPs to be tested, several templates were prepared. They differed as to the nucleotide present at the underlined position labeled N in the sequence. The template/primer (T/P) was adjusted to a final concentration of 25.0 nM in 50.0 μl of a mixture containing 25.0 mM Tris-HCl (pH 8.0), 75.0 mM KCl, 8.0 mM $MgCl_2$, 100.0 μg of bovine serum albumin per ml, and 10.0 mM CHAPS. The variable substrate (dNTP) was added at concentrations ranging from 1.25 to 40.0 μM. The reaction was initiated by the addition of RT to a final concentration of 0.1 nM. Reaction mixtures were incubated for 10 min at 37°C. The products were purified and then fractionated on a 15% polyacrylamide sequencing gel. The amount of primer (unextended primer and the reaction product of primer plus 1 nucleotide [primer + 1]) was determined by using the PhosphorImager system. The amount of primer + 1 was divided by the amount of total primer (primer and primer + 1) to determine the fraction of primer + 1 that was generated from the dNTP substrate in 10 min with the given amount of RT. The various kinetic values were calculated from these data.

RESULTS

AZTMP excision/extension assays. We analyzed the abilities of WT RT and three different mutants (the T215Y mutant, the M41L/T215Y double mutant, and the M41L/L210W/T215Y triple mutant) to use ATP to remove AZTMP from the 3′ end of the primer and to extend the primer to the end of the template strand. As described in Materials and Methods, AZTTP was present in these assay mixtures at a concentration of 1.0 μM so that during the extension of the primer strand, there was the possibility that AZTMP would be incorporated, rather than the normal dTTP substrate. The general protocol, which is shown in Fig. 2A, was designed to mimic what occurs during the conversion of the single-stranded viral RNA genome into double-stranded DNA in an infected cell. When the ATP was present at a concentration of 3.0 mM, WT HIV-1 RT inefficiently removed the AZTMP blocking group and extended the primer. This concentration of ATP is the standard concentration used for excision assays and was chosen

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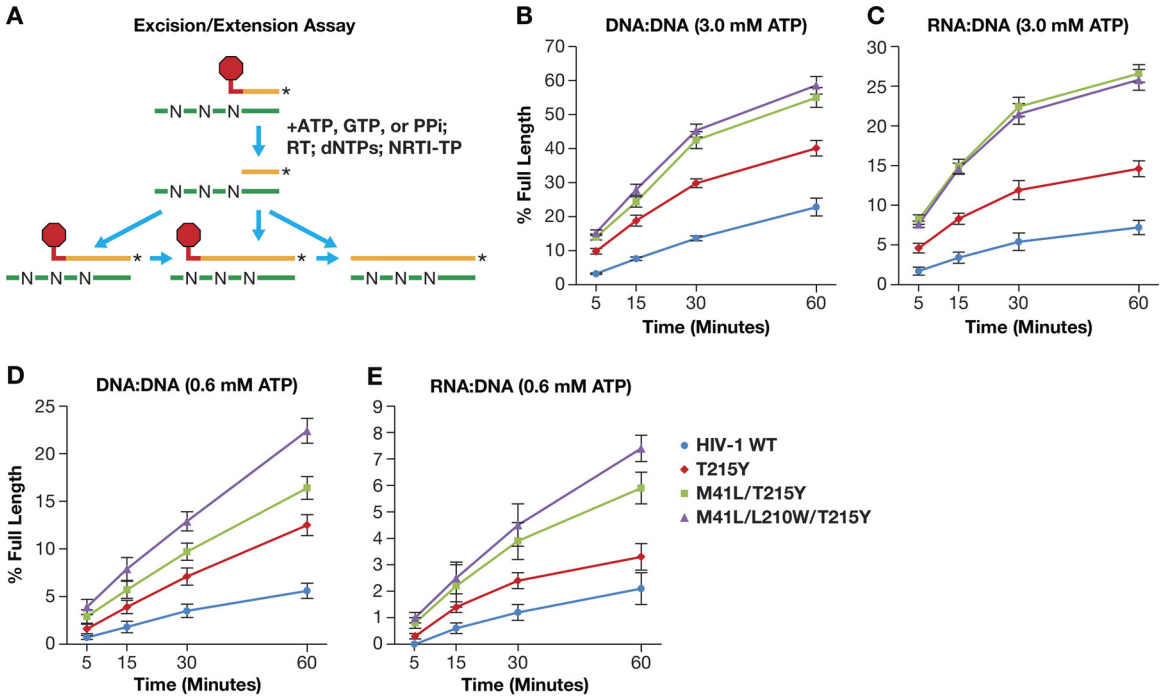

FIG 2 Comparison of the abilities of the various mutant RTs to excise AZTMP from the end of the blocked primer and extend the primer in the presence of AZTTP. (A) General outline of the experimental protocol. The template can be varied at the designated "N" site so that any NRTITP can be tested for its ability to be excised; however, in the experiments described in the text, the "N" is an "A" so that AZTMP will be incorporated. The asterisk indicates the presence of the ^{32}P end label on the primer. The end of the primer is blocked with the desired NRTIMP (in the experiments described in the text, the analog is AZTMP, which is represented by the stop sign). Either a DNA or an RNA template can be used in the reaction. The blocked template/primer is purified and used as a substrate for the mutant RTs in the presence of the indicated concentrations of ATP, 1.0 μM AZTTP, and 10.0 μM each dNTP at 37°C for the indicated amounts of time. The reactions are stopped, the template/primer is purified, and the products are fractionated by PAGE on a 15% gel. The amount of full-length product (extension of the primer to the end of the template) is measured by using the PhosphorImager system and is expressed as a percentage of the total amount of primer. (B and C) Ability of WT RT and the T215Y, M41L/T215Y, and M41L/L210W/T215Y mutants to excise AZTMP from the end of the primer using 3.0 mM ATP and extend the primer on either a DNA template (B) or an RNA template (C). (D and E) Excision/extension by these mutants with 0.6 mM ATP for a DNA template (D) and an RNA template (E).

because it is believed to be similar to the concentration that is present in dividing cells (15, 23, 24). The inefficient removal of AZTMP was evident in experiments done with either a DNA template (Fig. 2B) or an RNA template (Fig. 2C), although the amount of full-length product was smaller in the experiments done with the RNA template. The T215Y RT variant had an enhanced ability (~2-fold at the 60-min time point) to excise the AZTMP blocking group and fully extend the primer with either template, relative to the WT. Both the M41L/T215Y and M41L/L210W/T215Y mutant RTs were able to remove AZTMP and extend the primer even more efficiently than the RT carrying T215Y alone (Fig. 2B and C). However, in these assays, the M41L/L210W/T215Y mutant and the M41L/T215Y double mutant behaved similarly; there was no apparent advantage to having the L210W mutation present. HIV-1 can replicate in nondividing cells, where the concentrations of ribonucleoside triphosphates are lower than those in dividing cells, and based on the available data, we reduced the level of ATP to 0.6 mM and repeated the assays (15, 25) (Fig. 2D and E). In these assays, the M41L/L210W/T215Y mutant generated a higher level of full-length product in the excision/extension reaction, using a DNA template, than did either the M41L/T215Y double mutant (~8% more at 60 min) or the T215Y mutant (~2-fold more at 60 min). Results obtained by using an RNA template showed that the triple mutant was better at excision-extension than the T215Y mutant (slightly more than 2-fold more at 60 min). The results are less clear when the M41L/L210W/T215Y mutant is compared to the M41L/T215Y mutant. The level for the triple mutant was consistently higher, but the difference was small, only a few percentage points. It is not clear whether this difference would be significant for the replication of the virus. Thus, the addition of the L210W mutation appears to improve the ability of the M41L/L210W/T215Y mutant RT to use relatively low levels of ATP in the excision reaction. As shown in Fig. 1B, Y215 stacks with P217 in the absence of ATP and stacks with the adenine base of a bound ATP. In the T215Y mutant, the side chain of Y215 is likely to switch between the two orientations in the process of association and dissociation of ATP; however, the side chain of W210 (in the M41L/L210W/T215Y mutant) would stack with the side chain of Y215 and stabilize it in the ATP-bound conformation, which would provide better access for ATP and help it to bind when ATP is present at lower concentrations. Similar results were obtained in reactions in which UTP, GTP, or CTP was used as the excision substrate instead of ATP, although the overall level of excision varied depending on which NTP was used for the assays (see Fig. S1 to S3 in the supplemental material). The use of GTP generated approximately the same amount of full-length product as that with ATP; CTP and UTP tended to be less effective at producing the full-length product. This may reflect the fact that the nucleobases of ATP and GTP have two aromatic rings, while CTP and UTP have one. The interaction of the aromatic side

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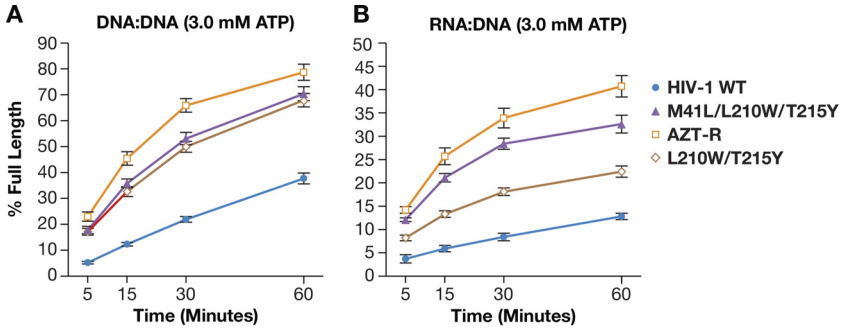

FIG 3 Comparison of the abilities of additional mutant RTs to excise AZTMP from the end of the blocked primer and extend the primer in the presence of AZTTP. Excision/extension reactions were carried out by WT RT and the L210W/T215Y, M41L/L210W/T215Y, and AZT-R (M41L/D67N/K70R/T215Y/K219Q) mutants on either a DNA (A) or an RNA (B) template, as described in the legend of Fig. 2 and in Materials and Methods.

chain of a tyrosine at position 215 may be better with purines, which present a larger aromatic surface, than with pyrimidines.

To better understand the contribution of the secondary mutations, we constructed a mutant that does not contain M41L (L210W/T215Y); this allowed us to study the effects of these two mutations without the influence of M41L. We also included the AZT-resistant variant AZT-R (M41L/D67N/K70R/T215Y/K219Q), which we have used in previous studies and which was the mutant used to generate the RT structure discussed above. Although all of the mutations in AZT-R can contribute to AZT resistance (7–10), the particular combination of mutations present in AZT-R does not appear to be commonly selected in patients. A search of the Stanford Database for all viruses in which the RT carries T215Y shows that the M41L mutation is also present 83.2% of the time, D67N is present 48.3% of the time, K70R is present 12.8% of the time, and K219Q is present 5.3% of the time. However, there are other mutations found at position 219: K219R is present 6.8% of the time that T215Y is present, K219E is present 6.7% of the time, and K219N is present 8.9% of the time. Thus, viruses that contain the M41L, D67N, and T215Y mutations are commonly seen in patients; however, viruses that also carry the two additional mutations present in the AZT-R RT are less common. The behavior of these new mutants was compared to those of WT RT and the M41L/L210W/T215Y triple mutant. As shown in Fig. 3A, the L210W/T215Y double mutant was equivalent, in the excision extension assay, to the M41L/L210W/T215Y triple mutant with a DNA:DNA template/primer, showing that the L210W mutation can enhance the ability of the T215Y mutant RT to excise AZTMP. However, with an RNA:DNA template/primer, the L210W/T215Y double mutant RT was not much better in the excision/extension assay (Fig. 3B) than an RT carrying T215Y alone (Fig. 2C). For both templates, AZT-R had the highest level of excision/extension activity (Fig. 3).

There have been some claims that for this group of mutations, AZT resistance involves a reduction in the ability of the mutant RT to incorporate AZTTP (26, 27). To confirm that excision was the only resistance pathway for these mutants, the WT and T215Y, M41L/T215Y, and M41L/L210W/T215Y mutant RTs were tested for their ability to incorporate AZTTP. Both an RNA template and a DNA template were tested. The DNA template is single-stranded M13mp18 DNA, and the RNA template contains sequences from the polypurine track, long terminal repeat, and primer binding site (PPT-LTR-PBS) of HIV-1. A labeled primer was extended in the presence of all four dNTPs and various concentrations of AZTTP. The susceptibilities of all of the RTs to inhibition by AZTTP were similar for both templates, indicating that none of the mutations had a significant effect on the ability of RT to incorporate AZTTP (Fig. 4A and B).

Polymerase assays. To determine how the various mutations affected the enzymatic activities of the WT and mutant RTs, several assays were performed. Because resting cells generally have lower dNTP levels than do actively growing cells (15, 25), the ability of the RTs to incorporate relatively low concentrations of dNTPs was tested by using both a DNA template and an RNA template. When the template was DNA, the T215Y RT mutant was less able to extend the primer than was WT RT. The defect in extension was apparent at all of the concentrations of dNTPs that were tested (Fig. 5A). The M41L/T215Y double mutant was better able to extend the primer than was the T215Y mutant but was still somewhat defective compared to WT RT. Thus, the addition of the M41L mutation appears to compensate to some degree for the reduced ability to incorporate dNTPs caused by the T215Y mutation. The addition of the L210W mutation to the double mutant (yielding the M41L/L210W/T215Y triple mutant) improved dNTP incorporation to a level similar to that of the WT (Fig. 5A). Similar results were obtained when the assays were repeated by

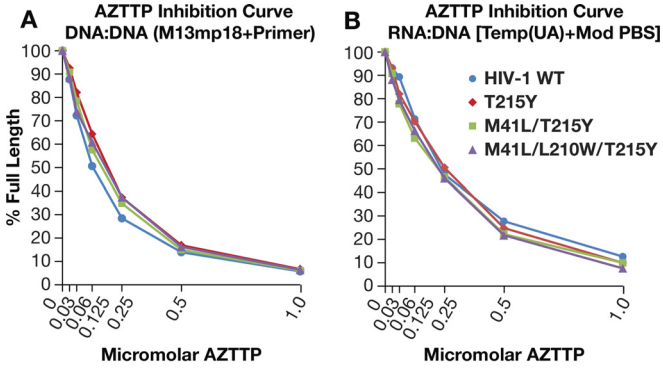

FIG 4 Ability of WT RT and the T215Y, M41L/T215Y, and M41L/L210W/T215Y mutants to incorporate AZTTP in the presence of normal dNTPs. The RTs were challenged with a range of AZTTP concentrations while the concentration of dNTPs was held constant. If the mutant RTs differ in their ability to bind and incorporate AZTTP, the amount of full-length product will decrease at a rate that differs from that of WT RT. Temp(UA) refers to the fact that the first 2 nucleotides in the template after the double-stranded region are uracil-adenine.

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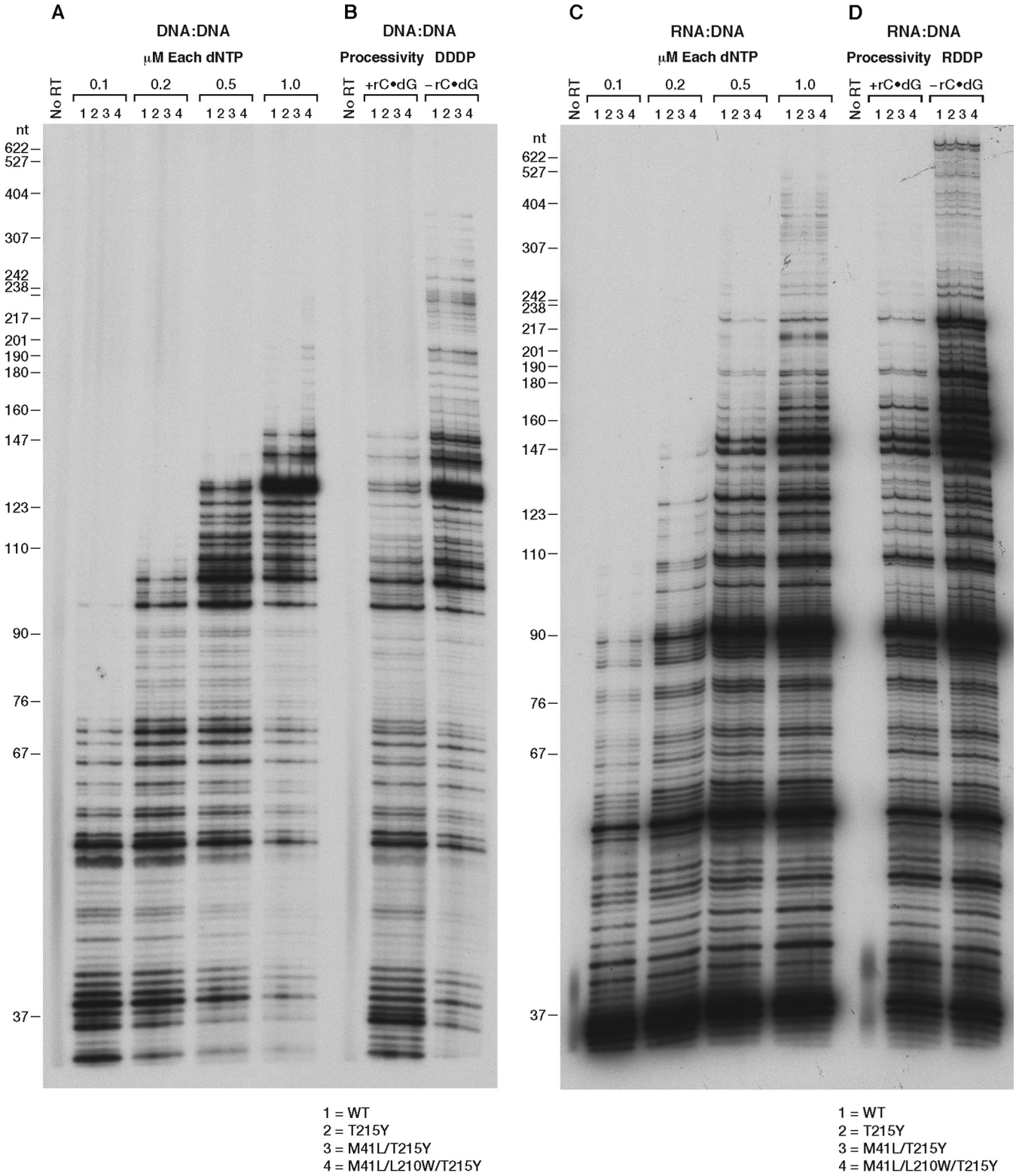

FIG 5 Comparison of the polymerase activities of the WT and mutant RTs. A 5′-end-labeled primer was annealed to single-stranded M13mp18 DNA (A, B, E, and F) or PPT-LTR-PBS RNA (C, D, G, and H). (A, C, E, and G) In the low-dNTP assay, RT was allowed to extend the labeled primer in the presence of suboptimal levels of dNTPs (0.1, 0.2, 0.5, and 1.0 μM each dNTP) for 15 min at 37°C. (B, D, F, and H) For the processivity assay, the 5′-end-labeled primer was annealed to single-stranded M13mp18 DNA or PPT-LTR-PBS RNA. RT was allowed to bind in the absence of dNTPs. dNTPs (20 μM each) and a "cold trap" [poly(rC)·oligo(dG)] were added to initiate the reaction, and the reaction mixtures were incubated at 37°C. The cold trap limits the polymerase reaction to one cycle of extension. The DDDP or RDDP reaction had no trap, and multiple rounds of binding and polymerization will occur. nt, nucleotide.

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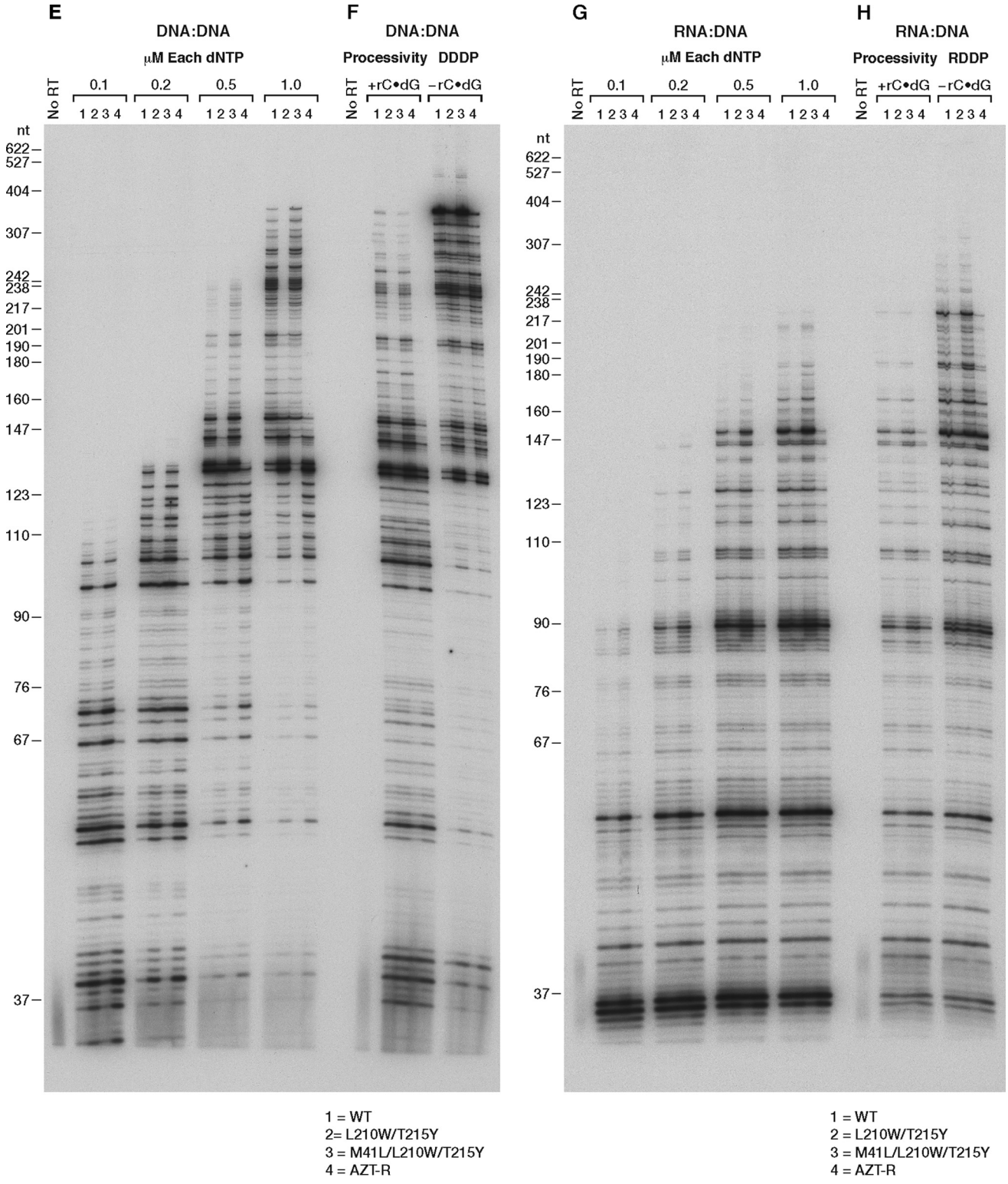

FIG 5 continued

using RNA as a template (Fig. 5C). The L210W/T215Y and AZT-R mutants showed much greater defects in polymerization than did the other RT variants, and both of these variants showed defects in polymerization with either a DNA or an RNA template (Fig. 5E and G).

Processivity assays and related DNA-dependent DNA polymerase (DDDP) assays were performed with higher levels of dNTPs (25.0 μM each) than those in the polymerization assays described above. These assays differ in that the processivity assays include a trap that limits RT to a single round of binding and

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polymerization. Once RT falls off the template/primer, it binds to the trap. The trap is omitted in the corresponding DDDP assays. With a DNA template, the T215Y mutant and the M41L/T215Y double mutant caused only a very modest defect in the processivity assay and may have had a modestly greater effect in the DDDP assay (Fig. 5B). These results suggest that a portion of the defect seen with the mutants is probably caused by an inability to bind and incorporate dNTPs efficiently when they are present at low to normal concentrations (Fig. 5A). The processivity assays were also done with an RNA template (Fig. 5D), and the results were similar. The mutations caused only modest defects in polymerase activity (Fig. 5D). A gel shift assay was used to compare the abilities of WT RT and the T215Y, M41L/T215Y, and M41L/L210W/T215Y mutant RTs to bind a DNA:DNA template/primer. The results showed that all of the RTs bound a template/primer with similar efficiencies (see Fig. S4 in the supplemental material). These data reinforce the idea that the primary problem with the polymerase activity of these mutant RTs is an impairment of their ability to bind and/or effectively incorporate dNTPs at low to normal concentrations. In contrast, the L210W/T215Y and AZT-R mutant RTs showed an obvious defect in the DDDP assay, the RNA-dependent DNA polymerase (RDDP) assay, and both processivity assays, which showed that relatively high concentrations of dNTPs were not enough to rescue the polymerization defects of these mutants (Fig. 5F and H). WT RT and the T215Y, M41L/T215Y, and M41L/L210W/T215Y mutants were also tested to determine if the mutant RTs had any defects in their RNase H activities. There were no significant difference in the RNase H activities of the RT mutants and the WT enzyme, suggesting that the RNase H active site is not affected by the alterations around the polymerase active site (see Fig. S5 in the supplemental material).

Kinetics. There are several difficulties that can be encountered when RT, or other DNA polymerases, is tested using steady-state kinetics. There are two substrates involved in the reaction, the template/primer and the dNTPs. The presence of excess template/primer can make the reaction pseudo-first order; i.e., excess template/primer acts to make the reaction appear to have only one reactant since the second substrate is not limiting. In addition, RT will encounter a wide variety of sequences when it copies either an RNA or a DNA template, and the efficiency with which RT incorporates and excises AZTMP varies depending on the sequence of the template (28). Finally, because RT copies single-stranded RNA and DNA template overhangs, the structure of the single-stranded portion of the template can affect the reaction. Despite these caveats, kinetic analysis using a template with a well-defined sequence and a variable concentration of one of the dNTPs can give useful insights into how the RT interacts with its substrates. The overall kinetics diagram and reaction substrates are shown in Fig. 6. Reactions were run under steady-state conditions. V_{max} and k_{cat} were derived from the same data; V_{max} is included to make the comparison of K_m values simpler (described below). V_{max} is the maximal amount of product (P), in this case a primer extended by 1 nucleotide, that can be generated per unit of time. k_{cat} is the amount of product generated in a unit of time per amount of enzyme (V_{max}/E_T) and is often referred to as the turnover number. Although the units are in seconds, the unit nanomolar P/nanomolar RT is implied, and k_{cat} refers to the number of reactions that each active site catalyzes per unit of time when the enzyme is fully saturated. When dTTP was the substrate, all of the mutants had a lower k_{cat} than that of WT RT, although the k_{cat} values for the M41L/T215Y and M41L/L210W/T215Y mutants were higher than that for the T215Y mutant (Table 1 and Fig. 6). The T215Y mutation also decreased the k_{cat} with dATP and dGTP. The k_{cat} values with dCTP are closer to those for WT RT. The values for the M41L/T215Y and M41L/L210W/T215Y mutants are similar to those for WT RT with dATP, dGTP, and dCTP. Therefore, under conditions in which the enzyme is saturated, only the T215Y mutant caused a significant decrease in the ability of RT to synthesize DNA; i.e., this mutant enzyme has a lower turnover rate than that of the WT, and the addition of the M41L/L201W mutation helps to rescue the efficiency of the polymerase.

K_m is the Michaelis constant and represents the amount of substrate (dNTP) needed to reach one-half V_{max}. Because K_m is a measure of the amount of substrate needed for efficient catalysis to occur, it is related to the ability of the enzyme to bind the substrate; thus, it is possible to have two RTs with similar V_{max} values that require different amounts of the substrate to reach the K_m. All of the mutants have V_{max} values that are lower than that of the WT for dTTP; in addition, their K_m values vary. The T215Y mutant has the highest K_m value, suggesting that this mutant needs more dTTP than does the WT to reach half-maximal velocity; the M41L/L210W/T215Y mutant also has a significantly higher K_m for dTTP than does the WT. The M41L/T215Y mutant has a K_m value for dTTP that is similar to that of the WT. The T215Y mutant also has an increased K_m value compared to that of WT RT for dCTP and dGTP. The M41L/T215Y and M41L/L210W/T215Y mutants have K_m values similar to those of the WT for dATP and dCTP, but their K_m values for dGTP are slightly increased.

The catalytic efficiency (k_{cat}/K_m ratio) of an enzyme is used as a measure of its overall ability to use the substrate. The catalytic efficiencies of the RTs have been calculated based on their ability to incorporate dNTPs, and the value for WT RT was set to 1.0. Overall, the T215Y RT mutant is the least efficient enzyme for all dNTP substrates (Table 1). The addition of M41L to either the T215Y mutant alone or the L210W/T215Y double mutant improved the catalytic efficiency of the enzyme. However, all of the mutants were less efficient than the WT, particularly with the dTTP substrate. These data indicate that the mutations interfere with the ability of the RT to bind and/or catalyze the incorporation of dNTPs; however, the effects were somewhat different not only for the different mutant RTs but also for the various dNTPs. In general, the presence of the M41L and L210W mutations compensated, to some extent, for the deleterious effects of the T215Y mutation on the polymerase activity of HIV-1 RT.

To see how well our data conform to what has been reported for patients, we examined the Stanford Database (http://hivdb.stanford.edu/) using an approach similar to that used by Yahi et al. (11). This was done by asking whether the amino acid substitution(s) of interest is present in the sequences of viruses isolated from patients. We first asked whether these mutations were strongly associated with a particular treatment history. We analyzed data reported between 2000 and 2005. Of the patients whose viruses had the T215Y mutation (and the drugs being used for therapy were known), 90.8% had been treated with AZT. Because stavudine (d4T) (which was used clinically but is no longer given to patients) also selects for similar excision mutations, we also checked for treatment with this drug (29). A total of 98.4% of patients who developed the T215Y mutation were treated with

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TABLE 1 Results obtained from kinetic reactions[a]

Substrate and enzyme	Mean V_{max} (nM dNTP min^{-1}) ± SD	Mean K_m (μM dNTP) ± SD	Mean k_{cat} (s^{-1}) ± SD	k_{cat}/K_m	Ratio
TTP					
WT	0.44 ± 0.04	0.92 ± 0.05	0.073 ± 0.007	0.079	1.0
T215Y	0.11 ± 0.02	2.12 ± 0.04	0.018 ± 0.003	0.009	0.11
41/215	0.16 ± 0.03	0.80 ± 0.03	0.027 ± 0.005	0.034	0.43
41/210/215	0.23 ± 0.01	1.53 ± 0.07	0.038 ± 0.002	0.025	0.32
dATP					
WT	0.68 ± 0.03	0.63 ± 0.02	0.113 ± 0.005	0.179	1.0
T215Y	0.43 ± 0.04	0.54 ± 0.03	0.072 ± 0.007	0.133	0.74
41/215	0.69 ± 0.05	0.61 ± 0.04	0.115 ± 0.008	0.189	1.06
41/210/215	0.68 ± 0.04	0.54 ± 0.03	0.113 ± 0.007	0.209	1.17
dCTP					
WT	0.31 ± 0.04	1.07 ± 0.07	0.052 ± 0.007	0.049	1.0
T215Y	0.29 ± 0.04	2.64 ± 0.10	0.048 ± 0.007	0.018	0.36
41/215	0.34 ± 0.02	1.23 ± 0.06	0.057 ± 0.003	0.046	0.93
41/210/215	0.30 ± 0.03	1.07 ± 0.05	0.050 ± 0.005	0.046	0.93
dGTP					
WT	0.34 ± 0.03	0.83 ± 0.03	0.057 ± 0.005	0.069	1.0
T215Y	0.22 ± 0.03	1.69 ± 0.04	0.037 ± 0.005	0.022	0.32
41/215	0.32 ± 0.01	1.55 ± 0.03	0.053 ± 0.002	0.034	0.49
41/210/215	0.33 ± 0.04	1.12 ± 0.04	0.055 ± 0.007	0.049	0.71

[a] Kinetic analysis was done as described in the legend of Fig. 6. The variable substrate [S] is the concentration of the dNTP being tested. WT refers to the wild-type RT, while T215Y contains only that mutation. 41/215 and 41/210/215 refer to RT mutations containing M41L/T215Y or M41L/L210W/T215Y, respectively. The product [primer + 1] is technically the concentration of PP_i released from the reaction, but this is equal to the amount of primer extended by one nucleotide (primer + 1). [E] is the amount of enzyme (0.1 nM RT). The use of the various rate constants is explained in the text. V_{max} is nanomolar primer + 1 per minute. K_m is the Michaelis-Menten constant in micromolar dNTP. The amount of S needed to obtain one-half V_{max} is calculated as follows: $K_m = (k_{-1} + k_2)/k_1$. k_{cat} is the "turnover number" per second, calculated as follows: $k_{cat} = V_{max}/[E]_t$. k_{cat} replaces the rate-limiting constant between ES and E plus P. The k_{cat}/K_m value is considered to be a measure of catalytic efficiency. Since these values can be hard to compare to each other, the last column (labeled "Ratio") has the catalytic efficiency set at 1.0 for the WT RT. The other catalytic efficiencies are normalized to this value.

either AZT or d4T. Thus, in the absence of d4T therapy, the excision mutation(s) is strongly associated with AZT therapy.

We also asked about the associations of the other amino acid substitutions that we analyzed. As shown in Table 2, in viral genomes (the initial search included all of the subtypes in the database) in which there was a T215Y mutation present (100% of sequences), an M41L mutation was also present 84.5% of the time, and an L210W mutation was present 62.3% of the time. For the subtype B genomes alone, an M41L mutation was also present 86.5% of the time, and L210W was present 66.4% of the time. This indicates that M41L is more important for the functioning of an AZT-resistant RT that carries the T215Y mutation than L210W. This matches what our assays showed: in the presence of a T215Y mutation, M41L increased both excision and polymerase activities, and L210W did not greatly increase excision and decreased polymerase activity when combined with T215Y. This suggests that for the L210W mutation to have a positive effect on RT, M41L might already have to be selected. Indeed, when both L210W and T215Y are present, M41L is also present 94.6% of the time. To be sure that these relationships were not affected by the HIV subtype, the analysis was done by using sequences of several different subtypes that were taken from the database individually. Although there was some variation, the frequencies with which the various mutations accompanied each other were similar for the various subtypes (see Table S1 in the supplemental material). Therefore, M41L appears to be the linchpin that allows for the selection of the L210W mutation, giving rise to the M41L/L210W/T215Y variant. M41L appears to increase the ability of the T215Y mutant to excise

FIG 6 (Right) Diagram of the kinetic reaction. The primer is labeled at the 5′ end and then annealed to the appropriate template, depending on which dNTP is being tested (in this diagram, dTTP is shown as being added where the template contains an "A"). (Left) Diagram indicating the various reactants and rate constants during the polymerization process. The reaction mixture contained 25.0 nM labeled template/primer (T/P) and 0.1 nM the appropriate RT. The reaction was initiated by the addition of the dNTP. Reactions were performed at 37°C for 10 min, the reactions were stopped, the T/P was purified, and the samples were fractionated on a 15% PAGE gel. The products were analyzed on a PhosphorImager instrument. The variable substrate (S) is the concentration of the dNTP being tested. P is technically the concentration of PP_i released from the reaction, but this is equal to the amount of primer extended by one nucleotide (primer + 1). The ratio of the primer band to the primer + 1 band was obtained from the PhosphorImager data and is presented as nanomolar P. E is the amount of enzyme (0.1 nM RT).

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TABLE 2 Presence of various mutations in RT[a]

Mutation that must be present	Other AZT resistance mutations			
	M41L	L210W	T215Y	T215F
M41L		58.0 (60.8)	75.2 (75.7)	13.1 (11.8)
L210W	93.0 (92.7)		89.1 (88.5)	4.2 (3.7)
T215Y	84.5 (86.5)	62.3 (66.4)		
L210W/T215Y	94.6 (94.7)			

[a] The presence of the various mutations in RT was determined by using the Stanford University HIV Drug Resistance Database (http://hivdb.stanford.edu/). The genotype treatment section was used to identify sequences that were selected by NRTI therapy. Samples where the treatment was unknown were excluded, since the presence of AZT could not be confirmed. The queries specified which amino acid(s) should be present in the sequences (left column). The percentages of other mutations present in these RT sequences were obtained from the database. The database was first examined for all subtypes and then just for subtype B (percentages in parentheses).

AZTMP-terminated primers, presumably by stabilizing the positioning of AZTMP at the N site.

DISCUSSION

We investigated the effects of the M41L, L210W, and T215Y mutations on the polymerase activity of HIV-1 RT and their effects on the ability of mutant enzymes to excise AZTMP. Our data indicate that subtle shifts in the ability of the mutant RTs to accomplish these two related tasks govern which combinations of mutations are selected in the virus in response to AZT therapy. There must be a balance between the ability of RT mutations to increase the binding of ATP for the excision reaction and any potentially negative effects that these mutations would have on the normal polymerization activity of the RT. As we reported previously, the T215Y mutation increases the ability of HIV-1 RT to excise AZTMP from the 3′ end of the primer using ATP as a pyrophosphate donor; however, at the same time, the T215Y mutation decreases the ability of RT to use low to moderate concentrations of dNTPs to carry out normal polymerization (17). At first glance, these results are confusing, since both reactions use the same polymerase active site. A possible resolution of this apparent paradox lies in the fact that the two reactions use different substrates, which is essential if the mutant enzymes are going to have an increase in their ability to excise AZTMP relative to their ability to incorporate AZTTP: primer-AZTMP + ATP → primer-OH + ATP-PP-AZTMP, and primer-OH + dNTP/AZTTP → primer-dNMP/AZTMP + PP_i.

We performed a biochemical analysis to dissect the effects of the M41L, L210W, and T215Y mutations on the two reactions. From the structure, it is clear that the primary role of the T215Y mutation is to provide a binding site for the adenosine ring of ATP. The improved ATP binding significantly increases the excision reaction (Fig. 1A); our excision assays confirm this result. However, it is also clear from our polymerase assays that T215Y has a deleterious effect on the normal polymerization reaction; why this would happen is not clear from the reported structures. We previously proposed that in order for the mutant RTs to be more effective in viral replication, the M41L and L210W mutations reduce the deleterious effects of T215Y on the polymerase activity of RT without decreasing its ability to excise AZTMP from the primer. The biochemical studies that we report here suggest that these secondary mutations increase the ability of RTs carrying the T215Y mutation to excise AZTMP. The initial excision reactions were performed by using 3.0 mM ATP. This concentration has served as the benchmark by many of the groups who have performed AZTMP excision assays because it is considered to be the physiological concentration of ATP in dividing cells (15, 18, 23, 25). However, this might not be the concentration that the RT sees during polymerization in some infected cells. Several studies suggested that the presence of the L210W mutation in RT causes a decrease in AZT susceptibility (7, 11, 12, 21, 30). However, in our experiments, when the ATP concentration was 3.0 mM, the triple mutant containing L210W was not significantly better in the excision/extension assay than the M41L/T215Y mutant. We saw differences in the excision/extension assay for the triple mutant compared to the double mutant when the ATP concentration was reduced (0.6 mM). This suggests that the levels of ATP that the RT encounters in some infected cells may not be as high as expected (3.0 mM). An obvious possibility is that there is a significant amount of HIV replication taking place in nondividing T cells or in other cells where the ATP concentration is <3 mM (15, 18, 23, 25). If so, the presence of the L210W mutation could allow more efficient binding of ATP at lower concentrations. It is also clear that the presence of the M41L mutation improves the polymerization ability of an RT carrying the L210W mutation and that L210W can improve the polymerization ability of an RT containing T215Y but, interestingly, only when M41L is also present (Fig. 5A and B). The mechanism by which the M41L and L210W mutations accomplish this is unknown. Kinetic analysis of the different RTs provides some insights into how the mutations affect the process of polymerization (Table 1). A general scheme for an enzyme-catalyzed reaction can be defined as

$$E + S \underset{K_{-1}}{\overset{K_1}{\rightleftarrows}} ES \xrightarrow{K_2} EP \xrightarrow{K_3} E + P$$

In the presence of an excess of the nucleic acid substrate, which was the case in our experiments, the enzyme (E) is RT, the variable substrate (S) is the dNTP being tested, and the product (P) is the dNMP joined to the primer. As described above, K_m represents the amount of substrate (dNTP) needed to reach one-half V_{max}. It can also be considered a ratio of all the rate constants involved in enzyme-substrate (ES) formation: $K_m = (k_{-1} + k_2)/k_1$. Therefore, mutations that decrease the ability of RT to bind the dNTP substrates (k_1 and k_{-1}) and/or how well the RT catalyzes the polymerization step (k_2) will increase the K_m, indicating that the mutation (or mutations) has a deleterious effect on RT. For most of the reactions that we ran, the T215Y mutant had an elevated K_m compared to those of the other RTs, indicating that this mutant RT has a decreased ability to bind dNTPs and/or a decreased ability to polymerize. For the most part, the M41L/T215Y and M41L/L210W/T215Y mutants have lower K_m values than does the T215Y mutant alone; they are closer to the WT in this regard. k_{cat} can be used to determine if the catalytic reaction is altered (binding of dNTPs might still be affected, but this calculation allows examination of polymerization itself). In the reaction described above, k_{cat} is usually equivalent to k_2, which is the rate constant of the catalytic step. In the polymerization reaction, the T215Y mutant has a k_{cat} value significantly below that of WT RT, indicating that this mutant has a problem with catalysis.

Although it is clear how the T215Y mutation helps RT bind ATP to improve excision, modeling and structural analyses have not offered any obvious insights into how T215Y impairs the polymerase activity of mutated RT. The T215Y mutation is ~9.0 Å from the polymerase active site. It is also ~9 Å away from the

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γ-phosphate of a bound dNTP (Fig. 1A) and does not appear to be in a position to interfere with dNTP binding. L210W enhances the effects of the T215Y mutation in the excision reaction and reduces the negative impact of T215Y on the polymerization reaction. Several models have been proposed for the role(s) of M41L and L210W in the excision reaction; however, none of them have significant experimental support. In our assays, the addition of L210W to the T215Y mutant reduced polymerization activity relative to that with T215Y alone, and compared to T215Y, the double mutant had a similar level of excision activity. Although it is relatively easy to envision an interaction between L210W and T215Y which could affect the position of the side chain of T215Y (2), which might enhance excision, it is much less clear how this might affect (enhance) polymerization.

Similarly, it is not clear how the addition of the M41L mutation, at a distance of 13 Å from T215Y site and 14 Å from the polymerase active site, improves the ability of the T215Y mutant to carry out either polymerization or AZTMP excision (Fig. 1A and C). It is not known whether the addition of M41L would affect the position of T215Y and, if it did, what the effect would be. In the apo-AZT-R structure, the addition of M41L does not appear to alter the position or orientation of Y215, nor does L41 seem to alter the position of any amino acid, even the putative target F116 (Fig. 1C).

It is possible that the T215Y mutation indirectly distorts the polymerase active site in a way that impairs the binding and incorporation of dNTPs in the polymerase reaction. However, because both the polymerization reaction and the excision reaction directly involve the polymerase active site, any distortion that impairs the polymerase activity is likely to also impair excision. Y215 is relatively far from the polymerase active site; the nucleobase of ATP (the moiety with which amino acid Y at position 215 is known to interact) is 9.0 Å away from the catalytic triad (D110, D185, and D186) (Fig. 1A). This means that the T215Y mutation should be able to have a much greater effect on ATP binding than on the polymerase active site, which is why it causes the increased excision that underlies AZT resistance. However, the T215Y mutation, by itself, has a significant negative impact on polymerase activity. It is possible that if the T215Y mutation has an indirect effect on the polymerase active site, M41L serves to ameliorate any distortions caused by T215Y and, perhaps, also enhances the binding and/or positioning of the AZTMP moiety on the end of the primer at the N site. Both of these roles could also enhance excision. In this scenario, the L210W mutation could potentially affect the positioning of T215Y through aromatic interactions of the amino acid side chains; this could, perhaps, relieve distortions at the active site caused by the T215Y mutation. Our data suggest that M41L must be present for the L210W mutation to be effective. The structure of an unliganded HIV-1 RT containing the D67N/K70R/T215F/K219Q mutation shows some changes in the positions of the three aspartate amino acids that coordinate the two Mg^{2+} ions at the polymerase active site (31). However, this structure was solved by using an unliganded form of RT that had neither a bound nucleic acid nor an incoming dNTP. It is not clear whether the distortions seen in this unliganded structure would have any impact on dNTP binding. Structures of RTs carrying the T215Y mutation, either alone or with M41L, did not show any distortion at the polymerase active site (32). Again, no dNTPs or nucleic acids were present in these structures. RT structures containing the T215Y mutation, with a double-stranded DNA template/primer and either with or without the product of the excision reaction (AZTppppA) bound at the active site, did not show any major changes in the polymerase active site (Fig. 1A) (2). At this point, a model in which the M41L and L210W mutations correct a distortion of the polymerase active site caused by T215Y is not strongly supported by the available structures.

We are considering a mechanism for how the L210W and M41L mutations might exert their effects, which might not be detected by crystallographic analysis. Previous studies of protease inhibitor (PI) resistance mutations in HIV-1 PR suggested that some PR mutations contribute to the flexibility of the hydrophobic core of the enzyme, a mechanism that has been called hydrophobic sliding (33, 34). In this mechanism, some of the resistance mutations that reduce the susceptibility of PR to PIs allow some of the hydrophobic core residues in PR to exchange interactions, allowing the residues (and the enzyme) more conformational flexibility, which in turn affects the processing of the cleavage sites in the HIV-1 polyproteins and reduces the binding of PIs that prevent these essential cleavages (33, 34).

Many of the primary drug resistance mutations found in HIV-1 give a modest to robust level of resistance, but resistance oftentimes comes at a cost to the replicative fitness of the virus. Secondary mutations can increase the fitness of the virus, increase the level of resistance, or both. For the secondary AZT resistance mutations that we tested, it appears that they can do both, based on our *in vitro* analysis of their effects on purified RT in assays that measure various aspects of the polymerase activity of RT and its ability to excise AZT and complete a short DNA product (using the excision/extension assay, which is designed to measure the susceptibility of RT and, by extension, the virus to inhibition by AZTTP). Moreover, in excision/extension assays, some of the secondary mutations had a greater impact with relatively low levels of ATP (0.6 mM) than at the concentration that is normally used for assays of this type (3.0 mM). As discussed above, the actual concentration of ATP in the cells in which the virus normally replicates in patients is not known; however, the fact that the secondary mutations appear to have an impact on excision at the lower ATP level suggests that, in at least some of the infected cells, the ATP concentration in the environment in which RT normally operates is probably <3.0 mM. This might be because an important fraction of viral replication occurs in quiescent cells, because the ATP in the cell may not have unfettered access to the RT that is present in the reverse transcriptase complex, or both.

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